IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In re Application of: | | § | |
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| Serial No.: | 09/881,721 | § | |
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| Filed: | June 18, 2001 | § | Group Art Unit: 1644 |
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| For: | METHODS OF UTILIZING CULTURED CULTURED HEMATOPOIETIC PROGENITOR CELLS FORINDUCING IMMUNOLOGICAL TOLERANCE | § | § |
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| | | § | Attorney |
| | | § | Docket: 01/21720 |
| Examiner: Mikhail A. Belyavskyi | | § | |

Commissioner for Patents
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## DECLARATION OF PROF. YAIR REISNER UNDER 37 CFR 1.132

I am presently employed as researcher at the Weizmann Institute of Science, Department of Immunology, where I am a Full Professor. I received my Ph.D. degree from The Weizmann Institute of Science in 1980, worked as a post-doctoral fellow at Memorial Sloan Kettering Cancer Center, New York and was a visiting Professor at Sloan Kettering Cancer Center, New York; Columbia University, New York and Dana Farber Cancer Institute, Harvard University Boston. A *curriculum vitae* is enclosed.

My research focuses on induction of tolerance for non-syngeneic transplants. . Since the beginning of my career, I have published 158 scientific articles in highly regarded journals and books, and have presented my achievements at many international scientific conferences.

I am a member of The American Society of Hematology Scientific Committee

on Transplantation Biology, and of The Immunology Working Party European Bone Marrow Transplantation (EBMT) Society, and have received a number of awards (see attached CV). I am an Associate Editor of The Journal of Experimental Hematology and serve on various editorial boards (see attached CV).

I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official Actions issued with respect to the above-identified application.

In this Official Action, the Examiner has rejected claims 1-2, 5, 7, 9-14, 16-17, 19-21 and 46-69 under 35 U.S.C. § 103(a) based on the contention that these claims are rendered obvious by U.S. Patent No. 5,806,529 in view of Bachar-Lustig *et al.*, or Mobest *et al.*, or Vavrova *et al.*

To the best of my knowledge, at the time the invention was made:

(i) it had never been documented that a human donor-derived cultured CD34+ cell population composed of at least 83.5 percent myeloid cells could possess enhanced veto activity (i.e. suppression of non-syngeneic cytotoxic T-lymphocyte precursors specific for antigens of the cultured cell population) per cell relative to a non-cultured human donor-derived CD34+ cell population; and

(ii) no transplantation method had ever been documented involving administering to a recipient both a non-syngeneic cultured human donor-derived CD34+ cell population composed of at least 83.5 percent myeloid cells, and a donor-derived graft.

As such, instant independent claims 1 and 12, and all claims dependent therefrom, are not rendered obvious under 35 U.S.C. § 103(a) by the teachings of U.S. Patent No. 5,806,529 in view of Bachar-Lustig *et al.*, or Mobest *et al.*, or Vavrova *et al.* because the combination of these documents fails to teach:

(i) that a human donor-derived cultured CD34+ cell population composed of at least 83.5 percent myeloid cells possesses veto activity which is enhanced per cell relative to a non-cultured human donor-derived CD34+ cell population;

(ii) any transplantation method involving administering to a recipient both a

non-syngeneic human donor-derived cultured CD34+ cell population composed of at least 83.5 percent myeloid cells, and a donor-derived graft for inducing graft tolerance; and/or

(iii) any potential graft rejection prevention benefit of administering to a recipient of a non-syngeneic donor-derived graft a human donor-derived cultured CD34+ cell population composed of at least 83.5 percent myeloid cells.

Additionally, during the interview conducted September 14, 2004, the Examiner verbally contended that the present invention is obvious in view of prior art teaching co-administration of donor-derived stem cells with cultured differentiated donor-derived cells to an allogeneic host for treatment of cytopenia in such a host. I have examined the prior art to which I believe the Examiner was referring to (refer to enclosed review article of Bock *et al.*) but have not found same to anticipate or render the present invention obvious. With respect to tolerance-inducing cells, at page 118, last two sentences of second paragraph, Bock *et al.* teach that it may be possible to co-transplant with PBPC, bone marrow-derived cells that can facilitate engraftment across immunological barriers taught by Kaufman *et al.* (refer to enclosed abstract). However, Kaufmann *et al.* teach that such cells are CD8+, CD3+, CD45R+, Thy1+, class IIdim/intermediate, TCR-negative cells; and that they differ significantly from other characterized lineages. Since, the cells taught by Kaufman *et al.* are clearly lymphoid in character by virtue of expressing the pan-T-cell marker Thy1, Kaufman *et al.* and Bock *et al.*, in fact teach away from using the cultured cells of the present invention which are composed of at least 83.5 percent myeloid cells for induction of graft tolerance. In distinct contrast, with respect to co-transplantation of donor-derived stem cells with differentiated cultured allogeneic donor-derived stem cells, Bock *et al.* consistently and exclusively teach that such co-transplantation is for prevention/correction of cytopenia, and at no point teach that such co-transplantation can be used for enhancing allogeneic graft tolerance in a host. For example, at page 118, second paragraph, Bock *et al.* teach that administration of a megadose of donor stem cells to an allogeneic recipient can be used to overcome immune barriers, and at page 119, first paragraph, last two sentences, teach that

administration of differentiated allogeneic donor-derived cultured stem cells is used for cytopenia treatment, even when immune barriers are successfully overcome via administration of a stem cell megadose. As such, Bock *et al.* clearly distinguish the tolerance-inducing capacity of undifferentiated stem cells from the reconstitution capacity of cultured/differentiated stem cells. In clear, unambiguous support of this distinction of the prior art, Bock *et al.* at page 118, last paragraph, list the potential utilities of administering to a recipient cultured/differentiated stem cells as being: (i) prevention/treatment of cytopenia; (ii) anti-tumor vaccination; and (iii) genetic therapy, all of which, however, without any mention of a potential utility for achieving prevention of graft rejection, even though the need for achieving prevention of graft rejection is discussed in the sentence immediately preceding this paragraph.

Hence, the prior art clearly neither teaches nor renders obvious the teaching of the present invention that administration of a non-syngeneic donor-derived cultured cell stem cell population composed of at least 83.5 percent myeloid cells, can be used for, and is moreover unexpectedly far more effective than, administration of non-cultured/undifferentiated stem cells for inducing tolerance to a donor-derived graft.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

October 21, 2004

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# Clinical use for expanded peripheral blood stem cells

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The increasing use of haematopoietic stem and progenitor cells from the peripheral blood (PBPC) to restore haematopoiesis following high-dose chemotherapy has widely propagated the development of techniques for the ex vivo manipulation of haematopoietic cells. In particular, protocols for the ex vivo expansion of PBPC have been developed for different clinical purposes. Quantitative expansion of PBPC may provide a successful strategy for tumour cell purging of autologous grafts, or may generate sufficient cell numbers for sequential transplantation protocols. Furthermore, allogeneic transplantation of megadoses of PBPC may enable us to overcome immunological barriers, and may substantially increase the number of suitable donors for an individual patient. Clinical applications also include the use of ex vivo generated, partially differentiated, post-progenitor cells, antigen presenting cells for immunotherapy of minimal residual disease, and ex vivo transduced haematopoietic cells as attractive vehicles for genetic therapy.

**Key words:** ex vivo expansion; peripheral blood progenitor cells; haematopoietic stem cells; stem cell transplantation; culture assays.

## INTRODUCTION

The increasing use of haematopoietic stem and progenitor cells from the peripheral blood (PBPC) to restore haematopoiesis following high-dose chemotherapy has widely propagated the development of techniques for the ex vivo manipulation of haematopoietic cells. Although replacement of bone marrow as the conventional

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source of stem cells by PBPC has brought about several clinical and logistical advantages, limitations still exist. These include infectious and haemorrhagic complications of cytopenia, inefficient PBPC collection and engraftment in heavily pretreated patients, or the relapse of disease due to graft-contaminating tumour cells. In the allogeneic setting, limitations may arise from engraftment failure or graft-versus-host disease, and the restricted availability of stem cell donors. As a consequence, improved transplantation strategies are needed. Recent progress in the characterization of haematopoietic stem cells and the development of techniques for the collection, procurement and manipulation of haematopoietic cells suggest that a wide use and broad spectrum of clinical applications for PBPC may be feasible in the near future, Among these studies, ex vivo expansion of PBPC represents a major focus of interest (see also Chapter 8).

Several concepts for the ex vivo expansion of PBPC for different clinical purposes have been developed (Figure 1). *Quantitative expansion* of PBPC may provide a successful strategy for purging contaminating tumour cells from grafts in the autologous transplantation setting. Efficient amplification of PBPC numbers may generate sufficient cells to allow sequential transplantation protocols. Also, expansion of haematopoietic cells from human umbilical cord blood may enhance its suitability for stem cell transplantation, which is currently limited to a body weight of less than 40 kg.[1] Allogeneic transplantation of megadoses of PBPC may even enable us to overcome immunological barriers, and may substantially increase the number of suitable donors for an individual patient.[2–5] This strategy may be possibly enhanced by the co-transplantation of cells that can facilitate engraftment across immunological barriers.[6]

*Differential expansion* and ex vivo generation of partialy differentiated progenitor (post-progenitor) cells may reduce the extent and duration of cytopenia. Also, *ex vivo produced immune cells (antigen presenting cells)* could be used for anti-tumour vaccination strategies against minimal residual malignant disease. Finally, *ex vivo transduced haematopoietic stem cells* represent attractive vehicles for genetic therapy.

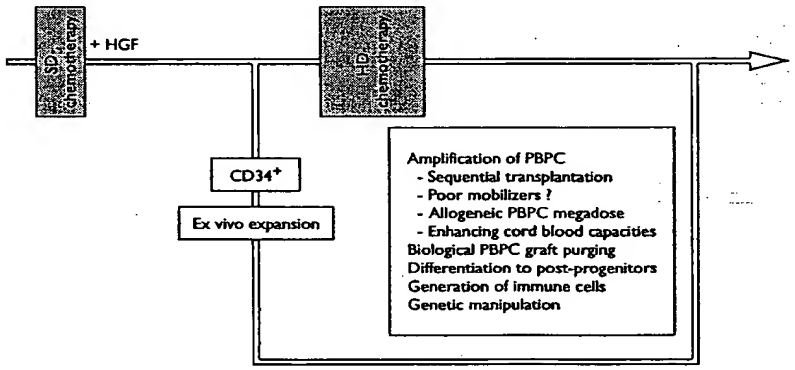


**Figure 1.** Potential clinical application of ex vivo expanded peripheral blood progenitor cells (PBPC). SD, standard dose; HD, high dose; $CD34^+$, CD34-selection of PBPC.

## REQUIREMENTS FOR EX VIVO EXPANDED STEM CELL GRAFTS

For an overall successful clinical transplantation, haematopoietic cellular protection must be provided in both the early as well as the late phases after transplantation. To provide long-term reconstitution of all lympho-haematopoietic lineages in a transplant recipient, pluripotent haematopoietic stem cells (PHSC, defined by their capacity for multi-lineage differentiation and self-renewal) have to be contained in the graft. This implies that PHSC have to be expanded or at least maintained in culture. However, early haematopoietic recovery appears to be mediated by various progenitor cell populations, which are different from PHSC and only provide short-term protection[7–10]; although the contribution of the different populations towards haematopoietic recovery is controversially discussed.[11–13] Thus, these progenitor subsets also have to be contained in the graft. However, even when megadoses of progenitor cells are transplanted, a cytopenic period of a few days cannot be avoided. Therefore, co-transplantation of a third cell population of so-called post-progenitor and precursor cells may be required in order to prevent cytopenia at all.

## PRECLINICAL RESULTS FOR THE DESIGN OF CLINICAL EXPANSION PROTOCOLS

Our group has been particularly interested in strategies for the purging of autologous PBPC grafts. Clinical studies using genetic marking have clearly demonstrated the existence of biologically active circulating malignant cells from solid tumours and haematological neoplasias[14], which can mediate relapse of malignant disease.[14,15] The concentration of contaminating tumour cells in the graft depends on the type and stage of malignant disease. Peripheral blood tumour cell concentration can be increased following chemotherapy and granulocyte colony-stimulating factor (G-CSF), and the time course of tumour cell mobilization can be similar to normal haematopoietic progenitors.[16] Co-mobilization is particularly high in metastatic breast cancer with bone marrow infiltration. Therefore, it appears to be useful to reduce the number of reinfused tumour cells by graft purging strategies.

Whereas tumour cell concentration was increased after a first cycle of chemotherapy, tumour cells have only very rarely been detected in peripheral blood after a second cycle of chemotherapy.[16] In order to obtain grafts with minimal contamination, we carry out two cycles of induction chemotherapy prior to progenitor cell harvest, and normal haematopoietic progenitor- and stem cells are positively selected by their CD34-antigen phenotype. Using CD34-enrichment, the tumour cell load of the grafts can be reduced by 2–4 logs compared to unseparated cells, provided that tumours are not carrying the CD34 antigen and are not themselves enriched. Haematopoietic reconstitution is not affected by CD34-selected compared to non-selected peripheral blood mononuclear cells.[17,18]

Differences in culture growth requirements between normal haematopoietic cells and malignant cells have been utilized for selective expansion strategies in order to biologically purge grafts from contaminating tumour cells.[19,20] The concept is to harvest only a small number of PBPC, which therefore include only a very small number of contaminating tumour cells (Figure 2). Then, the PBPC are specifically amplified ex vivo to numbers that are sufficient for transplantation, while the absolute number of tumour cells remains unchanged or ideally would even decrease. For this, conditions

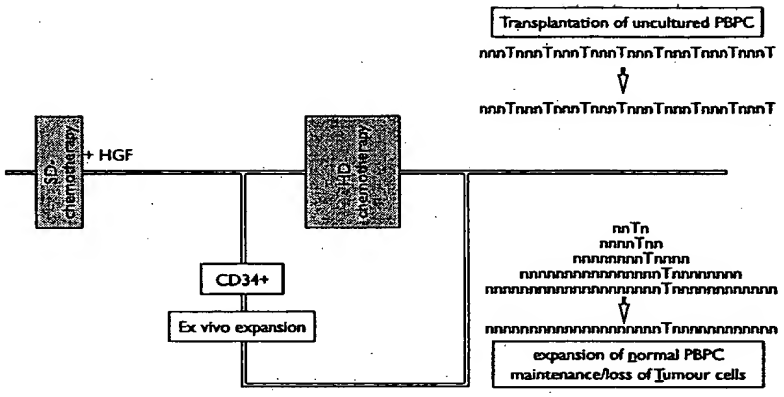


**Figure 2.** Biological PBPC graft purging by selective amplification of normal PBPC versus transplantation of uncultured PBPC. By ex vivo expansion of normal PBPC (n) without co-expansion of tumour cells (T) the number of tumour cells reinfused into the patient can be reduced by the expansion factor.

are required in which PBPC are expanded in vitro, but not the contaminating tumour cells.

To work out what conditions are required for this concept, our group has developed an experimental protocol of growing progenitor cells ex vivo from a relatively small volume of blood.[21] $CD34^+$ cells were cultured for up to 28 days using mainly (RPMI) medium, a cocktail of recombinant human colony-stimulating factors (CSF) and autologous plasma. All features of the experimental scale protocol also had to provide the option for clinical scale application. For this reason, a stromal cell layer was not included for feeding the cultured haematopoietic cells. Numerous conditions and combinations of haematopoietic growth factors have been tested in order to obtain optimal yields of colony-forming cells. A combination of recombinant human (rh) interleukin (IL)-1β, IL-3, IL-6, (stem cell factor (SCF)), and (erythropoietin (EPO)) provided the best results in terms of amplification of total mononuclear cells and number of colony-forming cells (CFC),[21] in which the total number of CFC are expanded about 100–200-fold and the maximum expansion was reached after a 2 week culture period.

In order to achieve sustained production of mature haematopoietic cells of different lineages after transplantation, it is critical to maintain primitive progenitor and stem cells with extensive proliferative capacity. As a potential indicator for the behaviour of early haematopoietic cells, we assayed long-term culture initiating cells (LTC-IC) at different time points in vitro, and maintenance of the number of LTC-IC could be demonstrated for 5–6 weeks.[22]

To test whether the number of contaminating tumour cells had been reduced in expanded grafts compared to uncultured cells, and whether tumour cells were co-expanded together with $CD34^+$ haematopoietic progenitor cells in vitro, cells derived from tumour cell lines were co-cultured with CD34-selected cells at varying ratios. Whereas total MNC were expanded about 100-fold, tumour cells from MCF-7, LXFS or primary RS-85 were maintained in culture, but did not increase in number during 2 weeks of co-culture.[23] Upon replating residual tumour cells into culture conditions that favour the growth of these tumour cell lines, tumour cells did regrow and thereby continued to express biological activity in vitro. Hence, exposure of the recipient

patient to graft-contaminating tumour cells is therefore reduced by clonogenic expansion.

Overall, the combination of in vivo purging, CD34-selection and biological purging ex vivo can reduce the number of reinfused tumour cells by 5–7 logs. Obviously, the more the normal and malignant populations differ in their growth requirements, the better a purging effect would be. Therefore, a more profound effect can be expected for solid tumours as opposed to haematological malignancies such as acute myeloid leukaemia.

## CLINICAL EXPERIENCE WITH EX VIVO EXPANDED CELLS

Based on these preclinical data, the ability of ex vivo-expanded progenitor cells to mediate haematopoietic reconstitution after high-dose therapy was tested in a clinical phase I/II study.[24] Ten patients with advanced cancer (non-small lung cancer (NSCL), $N = 5$; nasopharyngeal, $N = 1$; breast, $N = 1$; soft tissue sarcoma, $N = 1$; cancer of unknown primary, $N = 2$) who were eligible for high-dose chemotherapy were included in this phase I trial. The patients' age was between 25 and 57 years. Patients received two cycles of induction chemotherapy, at an interval of 3 weeks, consisting of etoposide (500 mg/m$^2$), ifosfamide (4000 mg/m$^2$), cisplatin (50 mg/m$^2$) and epirubicin (50 mg/m$^2$), a regimen previously shown to be active against a variety of cancers.[25] Twenty-four hours after the second cycle of chemotherapy, the patients received filgrastim (G-CSF; Neupogen, AMGEN) at a dose of 5 μg/kg of body weight subcutaneously to treat chemotherapy-associated neutropenia and to simultaneously mobilize PBPC. PBPC were collected in a single leukapheresis in which 6 litres of blood was processed.[26] CD34$^+$ cells were selected by immunoadsorption columns (Ceprate SC, CellPro).

For clinical transplantation, the starting population for culture consisted of a total of $15 \times 10^6$ cells after CD34-selection, which constituted about 10% of the leukapheresis yield. The cells were grown in 2% autologous plasma, rhSCF, rhIL-1β, rhIL-3, rhIL-6, and rhEPO for 12 days.[21] Non-adherent cells were collected, washed and resuspended for reinfusion. High-dose chemotherapy (etoposide 1500 mg/m$^2$, ifosfamide 12 g/m$^2$, carboplatin 750 mg/m$^2$, epirubicin 150 mg/m$^2$) was administered 3 weeks after the most recent induction treatment, and expanded progenitor cells were reinfused 24 hours after this therapy. No toxic side effects were observed after infusion of ex vivo-generated cells.[24]

The grafts contained a median of $12 \times 10^6$ expanded nucleated cells per kg, representing a 62-fold median increase during culture. Cultured cells gave rise to erythroid, granulocyte-macrophage, and multi-lineage colonies, with a 50-fold increase in clonogenic cells. A median of $1.2 \times 10^5$ CFC/kg were generated and transplanted. Four patients simultaneously received uncultured CD34$^+$ cells in addition to cells generated ex vivo, in order not to challenge haematopoietic recovery while possible toxic effects induced by cultured cells were still being evaluated.

Haematopoietic recovery was rapid in nine patients, while one patient died on day 14 due to neutropenic septicaemia. After transplantation of uncultured and expanded cells the median duration of a neutrophil count below 100 cells/mm$^3$ was 5 days (range, 5–7), and median time to platelet counts greater than 20 000 was 12 days (range, 11–15). When expanded cells only were transplanted, neutropenia lasted 6 days (range, 3–11), and thrombocytopenia 12 days (range, 11–15). No secondary cytopenic nadir was observed in any patient.

This study demonstrated for the first time the ability of autologous progenitor cells generated ex vivo to restore haematopoiesis after high-dose chemotherapy in cancer patients. The degree, time course and pattern of reconstitution was similar to historical control patients treated with either unseparated mononuclear cells or CD34-selected cells.[17] Since endogenous reconstitution might contribute to long-term haematopoiesis, no definite conclusions can be drawn with respect to the long-term capabilities of expanded cells (Figure 3, and see below).

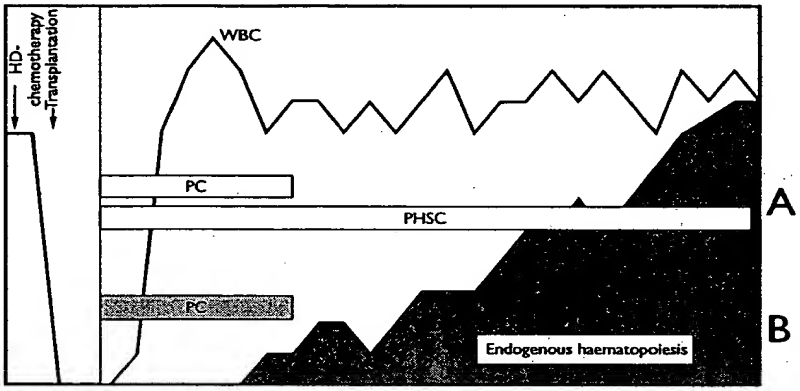


**Figure 3.** In the autologous transplantation setting, haematopoietic reconstitution could be mediated by transplanted progenitor cells (PC) and transplanted pluripotent haematopoietic stem cells (PHSC) or, alternatively, by endogenous haematopoiesis surviving high-dose chemotherapy plus PC. WBC, white blood cells.

In another trial by Alcorn et al[27], $CD34^+$ cells that have been expanded with the same cytokine combination but different growth conditions, have been transplanted together with unmanipulated cells. However, the IL-3 concentration of 10 ng/ml was significantly lower than that used by our group. The concentration of IL-3 may be of particular significance since IL-3 seemed to adversely affect the expansion of primitive haematopoietic cells in particular in some reports.[28]

In this phase I study, haematopoietic recovery of neutrophils and platelets was not affected by co-transplantation of expanded cells, and there was no toxicity of the cultured cells observed. Both clinical studies together indicate that current ex vivo expansion protocols do not further abrogate pancytopenia after chemotherapy. However, despite a low IL-3 concentration in the expansion cocktail, Alcorn et al[27] reported failure of sustained haematopoietic engraftment in three out of four patients that had been myeloablated and had received ex vivo expanded cells only.[29] Although in the patient with the lowest progenitor cell dose, engraftment did not occur either in the early or late phase after transplantation, short-term haematopoietic reconstitution was observed in the other three patients. In comparison to our study in solid tumour patients, the Alcorn study included patients with haematopoietic malignancies, which may contribute to the engraftment failures observed. It is apparent, however, that long-term haematopoiesis cannot be predicted unless maintenance or expansion of pluripotent haematopoietic stem cells (PHSC) has been demonstrated (see below).

## Long-term engraftment capacity of ex vivo expanded cells

In particular, for the transfer of ex vivo expansion protocols to complete myeloablative and allogeneic settings, it is critical to know whether long-term repopulating stem cells can be expanded or at least be maintained in culture. Clinical expansion studies in the autologous setting cannot answer this issue, since endogenous haematopoiesis can substantially contribute to haematopoiesis if high-dose chemotherapy is not completely myeloablative, and endogenous haematopoiesis cannot be differentiated or ruled out in an autologous situation (see Figure 3). However, secondary engraftment failures, as reported by Alcorn et al[27], indicates a loss of PHSC by their ex vivo manipulation.

To preclinically test ex vivo generated grafts for PHSC and primitive progenitor cells, assay systems are playing a major role for all ex vivo manipulation approaches.[30] In vitro assays such as the long term culture (LTC) assay, cobblestone area-forming cell (CAFC) assay, colony-forming unit blast (CFU-blast) assay and others have been established as test systems for primitive haematopoietic progenitors. Although some haematopoietic cells characterized in vitro have the multipotential and proliferative properties of PHSC, their capacity for long-term repopulation haematopoiesis in vivo, a hallmark of PHSC, has not been established. Therefore, the populations defined in vitro cannot be considered to be the equivalent of PHSC.[31] In animals, the properties of PHSC can be systematically analysed by multiple in vivo assays. Therefore, various strategies have been pursued to develop a chimeric animal model for human haematopoiesis. In fetal sheep and immunodeficient mice, the functions of human PHSC are reproduced, and long-term multilineage repopulation capacity and extensive proliferative potential have been demonstrated for xenografted human cells.[32–38] Thus, both models can be considered appropiate assays for the study of human stem cells and the development of stem cell therapies.[30]

Until very recently, ex vivo conditions neither allowed growth nor amplification of PHSC or primitive progenitors, and ex vivo expansion strategies consistently resulted in the loss of long-term repopulation capacity.[39,40] Recently, maintenance and modest amplification of primitive haematopoietic progenitors from umbilical cord blood have been demonstrated by a protocol that included flt-3 ligand, IL-3, IL-6, SCF and G-CSF, and repopulation was demonstrated for cultured cells in xenograft recipient animals.[41,42] Survival of the repopulating stem cells was limited to several days. None of these protocols, however, has been tested in a clinical study.

## Induction of partially differentiated haematopoietic cells

Even when excessive numbers of clonogenic progenitor cells had been transplanted in the past, it had not been possible to abrogate the cytopenic phase post-transplantation to less than 6–8 days. Nevertheless, transplantation of partially differentiated post-progenitor cells (myeloblasts, myelocytes, megakaryoblasts, megakaryocytes) could allow an immediate haematopoietic reconstitution since the time necessary for differentiation from primitive stem and progenitors to mature effector cells would be substantially reduced. The clinical success of these approaches mainly depends on two factors, the feasibility of ex vivo generation of sufficient cell numbers for transplantation, and the ability of the ex vivo generated post-progenitor cells to home, engraft and expand in vivo.

Ex vivo differentiation of $CD34^+$ PBPC preferentially into the myeloid lineage has been shown using IL-3 and G-CSF or GM-CSF.[43,44] Also, computerized analysis of

myeloid differentiation using multiple biological, logistical and methodological parameters suggested the feasibility of generating sufficient numbers of myeloid post-progenitors from $CD34^+$ PBPC on a clinical scale.[43a] However, the model suggests that large numbers of $CD34^+$ cells (approx. $7 \times 10^6$/kg) would be necessary to generate the minimum number of $6 \times 10^8$ myeloid post-progenitor cells/kg required for sustained neutrophil counts beyond 100/μl.

Williams et al[45] co-transplanted a mean total of $44 \times 10^6$ myeloid post-progenitor cells/kg. A tendency towards a very early engraftment was observed, which was dependent on the transfused cell number. Because of the small number of recruited patients, however, this effect will have to be confirmed, particularly by using the high numbers of post-progenitor cells suggested in our computer model.

IL-3, IL-6, IL-11 and in particular thrombopoietin are able to induce differentiation of early haematopoietic progenitors to megakaryocytes and megakaryoblasts.[46,47] Bertolini et al[48] generated $CD34^+/CD61^+$ cells, CFU-megakaryocytes, and $CD41^+$ megakaryocytic cells ex vivo in liquid culture with multiple cytokines, including thrombopoietin. Platelet transfusion requirements appeared to be lower than generally transfused, and two out of four recruited patients did not need platelet transfusions at all. In particular for the generation of post-progenitor cells, large-scale in vitro procedures for the generation of excessive cell numbers are essential for the success of these strategies.

## Ex vivo generation of immune cells

Minimal residual tumour cells can often induce relapse of malignant disease after high-dose chemotherapy and stem cell transplantation (SCT). Therefore, ex vivo generation of professional antigen presenting cells (dendritic cells (DC)) and their co-transplantation at a time of minimal tumour burden (soon after SCT) is of high therapeutical interest. DC are potent initiators of immune responses via T-lymphocytes, and play a major role in the priming and activation of cytotoxic T-cells (for reviews, see Peters et al[49]; van Schooten et al).[50]

Multiple protocols allow cytokine-induced ex vivo generation of DC from bone marrow, cord blood, or PBPC.[51–55] GM-CSF appears to be important for DC differentiation, and SCF for their quantitative yield. IL-4 can inhibit monocytic differentiation and also appears to be critical for a high cellular yield.[51,56] IL-4 and GM-CSF enhance antigen presentation capacities, which are reduced by tumour necrosis factor-α (TNF-α).[52–54,56] It is currently unknown which conditions serve best for the generation of functional DC for immunotherapy. Although DC can be effectively generated in serum- or plasma-containing media, the growth requirements for their generation in serum devoid of serum or plasma are poorly understood. Data from Strobl et al[57] and data from our own group[58] suggest that transforming growth factor-β is indispensable for DC growth in serum-free medium. Alternatively, functionally active DC can also be generated from blood monocytes even in the absence of GM-CSF.[56,59]

For the induction of tumour antigen presentation, ex vivo generated DC can either be pulsed in vitro with tumour-specific soluble antigens or peptides or transfected with complimentary DNA encoding for tumour-specific antigens. Ex vivo generated DC can be either used to generate tumour-specific cytotoxic T-cells ex vivo for subsequent transfusion or can be administered directly to patients for the induction of an in vivo tumour-specific immune response. It is currently unknown which techniques or DC characteristics are best for an optimized antigen processing and presentation

for immunotherapeutic purposes. Clinical studies using cancer vaccines are under way, and some have already demonstrated clinical efficacy.[60,61]

## SUMMARY AND PROSPECTS

Transplantation of autologous PBPC generated ex vivo can restore haematopoiesis after high-dose chemotherapy in cancer patients. A total number of $10 \times 10^6$ CD34-selected cells (one-tenth of the normal PBPC cell dose) prior to cytokine-induced ex vivo expansion yields sufficient progenitor cells to permit rapid and sustained haematopoietic recovery after high-dose chemotherapy in adults. No toxic side effects related to the cellular product have been observed. The degree of reconstitution is similar to historical control patients treated with either unseparated mononuclear cells or CD34-selected cells. Thus, the combination of ex vivo-epansion and CD34-enrichment of a limited number of PBPC reduces the number of tumour cells that are reinfused into the patient by 4–5 logs. Whether ex vivo expanded cells alone can be used in a complete myeloablative regimen will depend on the development of culture protocols that maintain or even expand PHSC.

Generation of partially differentiated progenitor cells may reduce pancytopenia following high-dose chemotherapy. Clinical benefit has not been demonstrated yet and will mainly depend on the homing capacity of ex vivo generated post-progenitor cells in the recipient environment. Recent progress in the ex vivo generation of large numbers of immune cells has led to novel immunotherapy approaches for tumours. The concepts of ex vivo generation of antigen presenting dendritic cells, vaccination and the induction of an immune response in vivo against tumour-associated antigens appears promising and is presently being studied. Altogether, the field of ex vivo manipulation of haematopoietic stem and progenitor cells is continuing to be a dynamic and challenging field, and involves many future clinical prospects.

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# Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells.

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Bone marrow transplantation is an accepted therapy for hematologic malignancies, aplastic anemia, metabolic disorders, and solid tumors. However, graft-versus-host disease (GVHD) and failure of engraftment have limited the widespread application of this technology to nonmalignant disease states. The use of purified bone marrow stem cells has been suggested as an approach to promote engraftment yet avoid GVHD. Although bone marrow stem cells, purified by cell sorting, engraft and repopulate lethally irradiated genetically identical recipients, they do not engraft in major histocompatibility complex (MHC)-disparate allogeneic recipients. We report for the first time the characterization of a novel cell population of donor bone marrow origin, separate from the hematopoietic stem cell, that facilitates engraftment of purified allogeneic bone marrow stem cells in an MHC-specific fashion without causing GVHD. Although 1,000 purified stem cells (c-kit+/Sca-1+/lineage-) reliably repopulate syngeneic mouse recipients, 10 times that number do not engraft in MHC-disparate allogeneic recipients. The addition of as few as 30,000 facilitating cells (CD8+/CD45R+/TCR-) is sufficient to permit engraftment of purified stem cells in MHC-disparate recipients. The cell surface phenotype of this purified cellular population differs significantly from other characterized lineages of lymphoid or myeloid origin. Based on multiparameter rare-events cell sorting, the facilitating fraction is CD8+, CD3+, CD45R+, Thy 1+, class IIdim/intermediate but alpha beta-TCR- and gamma delta-TCR-. This cellular population comprises approximately 0.4% of the total bone marrow and is separate from the hematopoietic stem cell. The coadministration of purified facilitating cells plus stem cells to optimize engraftment yet avoid GVHD may expand the potential application of bone marrow transplantation to disease states in which the morbidity and mortality associated with conventional BMT cannot be justified.

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